

HYPOTENSIVE RESUSCITATION OF CASUALTIES IN THE FAR-FORWARD COMBAT ENVIRONMENT: EFFECTS OF SELECT CRYSTALLOIDS AND COLLOIDS ON SIGNAL TRANSDUCTION MEDIATORS IN A SWINE MODEL OF SEVERE HEMORRHAGE

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1. BACKGROUND

Hemorrhage remains a major cause of death on the battlefield in conventional warfare (Bellamy, 1984). Current dogma dictates that early, adequate fluid resuscitation is crucial to reduce the mortality and morbidity associated with hemorrhagic shock. Yet, despite much research in the field and years of resuscitating thousands of patients, the optimal fluid and resuscitation strategy for the treatment of hemorrhagic hypovolemia remains unknown. However, with future combat strategies focused around the Future Force Warrior, greater dispersal of troops and fighting in urban settings and on non-linear battlefields, the likelihood of longer evacuation times for combat casualties is anticipated. As a consequence of these conditions and the logistic limitations of weight and cube, fluid resuscitation research within the Army's Combat Casualty Care Research Program has focused to investigate limited- or small-volume fluid resuscitation strategies, including permissive hypotension, in far-forward areas for the treatment of severe hemorrhage. The ultimate goals are to improve battlefield survival and to reduce or prevent early and late deleterious sequelae in the injured soldier. For the military the concept of hypotensive resuscitation, or fluid resuscitation to a blood pressure below pre-hemorrhage levels, currently seems to be a rational approach to compensate for the limited amount of fluid available on the battlefield to treat casualties, and to minimize the chance for rebleeding from penetrating injuries. In addition, studies in experimental animals have suggested that hypotensive resuscitation may improve survival from an uncontrolled hemorrhage (Capone et al, 1995; Stern et al, 2001).

The importance of restoring adequate tissue perfusion after hemorrhage to maximize recovery and minimize morbidity is self-evident. Nevertheless, a certain proportion of casualties who survive initially, go

on to develop organ failure, with a resultant poor outcome (Hardaway, 1982; Bellamy et al, 1986). Although the mechanisms for this failed resuscitation are poorly understood, evidence suggests that cytokines and other mediators of shock may be involved. Activation of protein tyrosine kinases, including the enzyme associated with the epidermal growth factor receptor (EGF-R), has been suggested to be an early event in the signal transduction pathways that lead to synthesis of these mediators (Dong et al, 1993; Marczin et al, 1993; Akarasereenont et al, 1996). Previous studies have shown that tyrosine kinase activity and expression of the EGF-R are induced by tissue injury (Tarnawski et al, 1992; Relan et al, 1995). Protein tyrosine kinases catalyze phosphorylation of tyrosine residues in proteins and have been associated with receptors of a number of growth factors, such as EGF and products of proto-oncogenes involved in the regulation of tissue growth, differentiation and repair (Humes et al, 1989; Cadena and Gill, 1992; Cantley et al, 1991).

Evidence also suggests that reactive oxygen species may be involved in the mechanisms through which tissue injury may stimulate protein tyrosine kinases (Suzuki et al, 1997). In addition, reactive oxygen species have been reported to regulate transcription factors involved in the synthesis of proinflammatory cytokines and other mediators of inflammation and the stress response (Li and Karin, 1999). For example, hydrogen peroxide can directly activate tyrosine kinase activity of the EGF-R in vitro, independent of the action of EGF (Gamou and Shimizu, 1995).

Since the gut has been implicated in mediating organ dysfunction in response to hemorrhage and shock (Magnotti et al, 1998; Wang et al, 1998), it is reasonable to speculate that small intestinal tyrosine kinase could be activated in response to hemorrhage and fluid resuscitation. Thus, the present study examined EGF-R

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activation and evidence of free radical generation in duodenum from pigs subjected to severe hemorrhage and resuscitated with lactated Ringer's (LR) as the standard of care, or the colloids, Hextend or PolyHeme. Hextend is a hetastarch in a balanced salt solution carried by the Special Operations Forces medics and PolyHeme is a human hemoglobin therapeutic with oxygen carrying capability under evaluation for use by the military in forward echelons of care. In addition, since the lung is often a target organ of injury associated with mediators from the gut (Sambol et al, 2000; Osband et al, 2004), EGF-R activation and free radical generation in lung was also evaluated to compare with the duodenum.

2. PROCEDURE

Immature female swine (n=8-10/group), weighing about 40 kg, were anesthetized, splenectomized and instrumented with arterial and venous catheters for hemodynamic and blood gas measurements, blood withdrawal and fluid infusion. After baseline recordings each pig was subjected to a controlled hemorrhage of 20 ml/kg over 4 min 40 sec that matched the blood loss profile of an uncontrolled hemorrhage (Fig. 1).

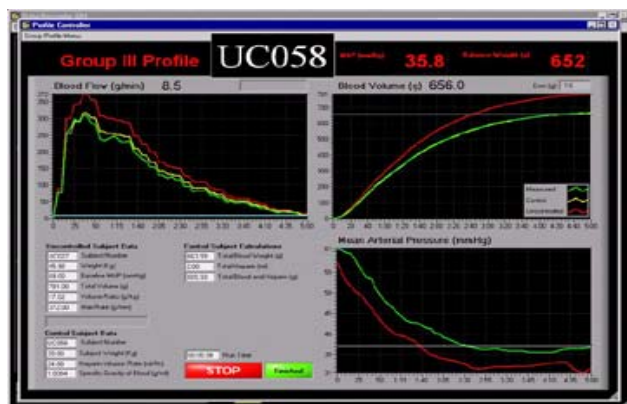


Fig. 1. Example of hemorrhage profile. Graphs depict pump speed (upper left), blood volume loss (upper right) and mean arterial blood pressure (bottom right). Focusing on upper right, red line depicts blood loss from an uncontrolled hemorrhage, yellow line is blood loss calculated for this specific experimental animal and green line overlapped on yellow shows the high degree of matching of the blood loss profile.

After 30 min the pig was subjected to a second hemorrhage of 8 ml/kg that followed the same blood loss profile. Fluid resuscitation, under a hypotensive resuscitation strategy, was begun during the second hemorrhage period and was continued to return and maintain systolic blood pressure to 80 mmHg, as necessary using a computer-controlled infusion pump program. LR and Hextend were infused at 1.5 ml/kg/min

and PolyHeme was infused at 1.0 ml/kg/min to minimize overshooting the target blood pressure. All animals were monitored for 3 hr after the start of resuscitation or until death. Hemodynamic and metabolic variables were monitored continuously throughout the experimental period. Blood samples were drawn at baseline, the end of the first hemorrhage, and at 15, 30, 60, 90, 120, 180 and 210 min or at death for determination of plasma lactate, hemoglobin and hematocrit by standard clinical chemistry techniques.

At the end of the experimental period, duodenal and lung samples were collected and frozen rapidly until analyzed. Tissues were solubilized in lysis buffer as previously described (Sondeen et al, 1999). The content of total and phosphorylated EGF-R were determined by immunoprecipitation followed by Western blot analysis using polyclonal antibody to EGF-R (UBI, Lake Placid, NY) and monoclonal antibody to phosphotyrosine (PY20; Transduction Laboratories, Lexington, KY), respectively. Other details of the procedures have been reported previously (Tureaud et al, 1997; Sondeen et al, 1999). Protein content was determined by a commercial kit (BioRad Laboratories, Richmond, CA). Evidence for lipid peroxidation or free radical generation in these tissues was determined by evaluating total antioxidant potential or the levels of thiobarbituric acid reactive substances (TBARS), 8-isoprostane, nitric oxide and glutathione. Total antioxidant potential, TBARS and glutathione were determined spectrophotometrically as previously described (Park et al, 2004). Nitric oxide and 8-isoprostane were quantitated by commercially available kits (Stressgen Biotechnologies Corp, Victoria, BC, Canada). Results were normalized to tissue protein concentrations. Hemodynamic and metabolic data were analyzed statistically by analysis of variance with repeated measures on time. Data from duodenum and lung were analyzed by analysis of variance and were compared to data obtained from 3 time control animals. Survival data were analyzed by Fisher's Exact test. Post-hoc comparisons used Tukey's test with Bonferroni correction. A $p < 0.05$ was considered significant.

3. RESULTS

The hemorrhage model employed in the present study was 100% lethal without fluid resuscitation. Fluid resuscitation resulted in survival rates of 8/10 in the LR group, 6/8 in the Hextend group and 8/10 in the PolyHeme group. These differences were not statistically different among the fluid groups.

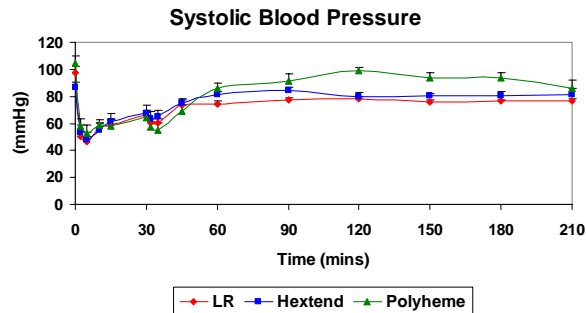


Fig. 2 Systolic blood pressure over time in pigs hemorrhaged at time 0 and at 30 min, and fluid resuscitated to a target blood pressure of 80 mmHg.

As shown in Fig 2, the initial hemorrhage reduced systolic blood pressure (SBP) to about 50 mmHg. The figure also illustrates some degree of autoresuscitation in the animals and the small drop in SBP in response to the second hemorrhage. After initial resuscitation, PolyHeme tended to maintain SBP a little above the target SBP of 80 mmHg, while LR and Hextend maintained SBP a little lower than the target pressure. To maintain SPB at or near the target blood pressure required over 3 times as much LR as both PolyHeme and Hextend (89 ± 11 ml/kg vs 24 ± 3 and 26 ± 6 ml/kg, respectively). Cardiac output (CO) fell over 50% in response to the initial hemorrhage and after some recovery, fell further in response to the second hemorrhage (Fig 3). Although the lowest CO compared with baseline was in the PolyHeme group, PolyHeme resuscitation actually improved CO over 2-fold after hemorrhage, whereas Hextend and LR improved CO about 1.7-fold (Fig 3). These differences were not statistically significant.

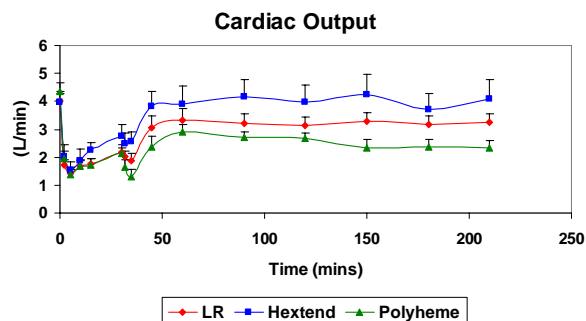


Fig. 3 Cardiac output in response to hemorrhage and fluid resuscitation.

Arterial base excess declined shortly after hemorrhage in all groups (Fig 4). After 60 min, the developing base deficit leveled off in all groups and was worse in the LR and PolyHeme groups than the Hextend group (Fig 4). Plasma lactate concentrations followed the developing base excess (Fig 5). The highest lactates developed in the PolyHeme group, but at the end of the 210 min experimental period, plasma lactates were

significantly higher than baseline, but there were no significant differences among groups (Fig 5).

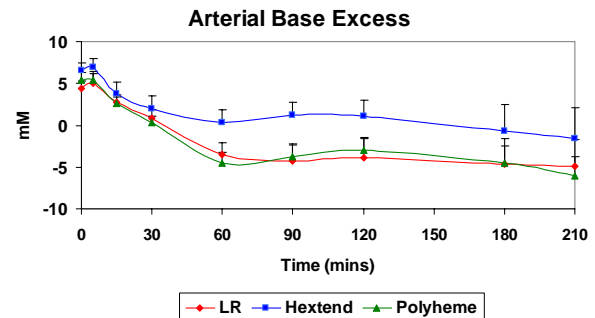


Fig. 4 Calculated arterial base excess in hemorrhaged and fluid resuscitated pigs.

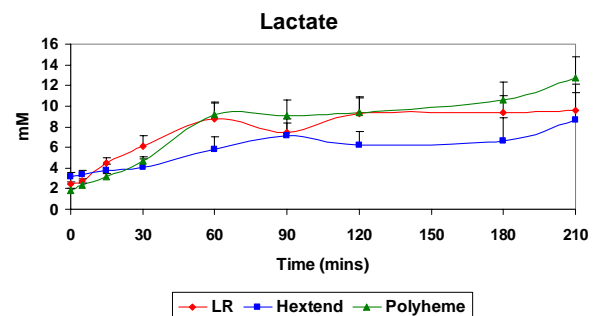


Fig. 5 Plasma lactate concentrations in response to hemorrhage and fluid resuscitation.

Biochemical analysis revealed that duodenal EGF-R levels were 26% lower in LR treated animals than in controls (Fig 6). In contrast, duodenal EGF-R concentrations in the PolyHeme and Hextend groups, were within 90% of control levels (Fig 6). In lung, EGF-R levels in LR and Hextend treated pigs were 51% and 44% lower than controls, respectively, while in the PolyHeme group, these levels were similar to controls (Fig 6). Phosphorylated EGF-R (p-EGF-R) levels in duodenum, representing EGF-R activation, were 27% lower than controls in the LR group (Fig 7). In the Hextend group these levels were similar to controls, whereas in the PolyHeme group p-EGF-R levels were about 80% of controls (Fig 7). In lung, p-EGF-R levels in LR and Hextend treated animals were significantly lower than control values, whereas these levels in PolyHeme treated animals were 47% higher than controls (Fig 7). Further interpretation of these data revealed that the proportion of phosphorylated EGF receptor in the duodenum was unchanged by hemorrhage and fluid resuscitation, whereas in lung, this proportion was about 38% higher in LR and PolyHeme-treated animals compared with controls.

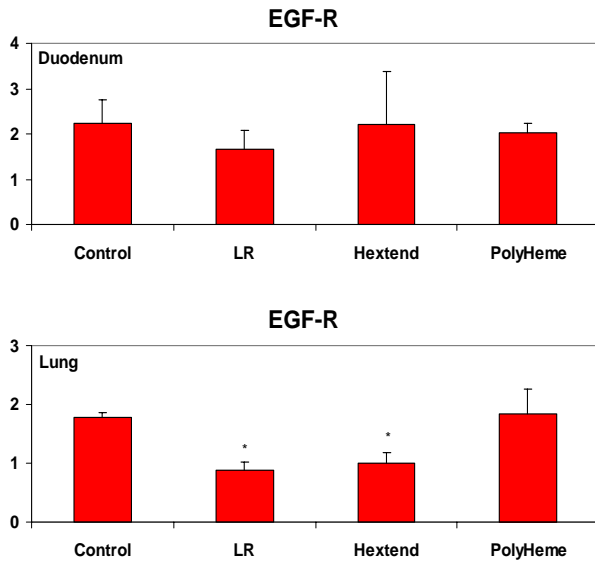


Fig. 6 Relative levels of the epidermal growth factor receptor in duodenum (top) and lung (bottom). $P < 0.05$ from controls

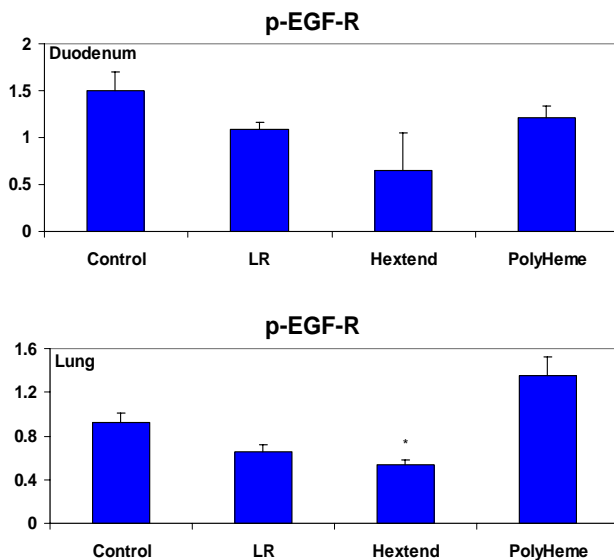


Fig. 7 Relative levels of the phosphorylated epidermal growth factor receptor in duodenum (top) and lung (bottom). * $P < 0.05$ from controls

No significant differences were observed among groups in duodenal TBARS, 8-isoprostane or nitric oxide levels (Table 1). Duodenal total antioxidant potential was significantly higher in the PolyHeme group compared with controls. However, duodenum glutathione (GSH) concentrations in the LR group were 36% lower than controls (Table 1), whereas duodenal GSH levels in Hextend and PolyHeme treated animals were about 15% higher than GSH concentrations from the LR group. For comparison, lung TBARS and total antioxidant potential

were nearly 2.5-fold higher and 32% lower, respectively, in LR-resuscitated animals than controls (Table 1). Lung TBARS concentrations were also significantly lower in the PolyHeme than LR group. Although GSH concentrations were 30% lower in the LR group than controls, large variability prevented the data from achieving statistical significance. In contrast, GSH concentrations in the Hextend and PolyHeme group were closer to control levels (Table 1). In addition, lung 8-isoprostane and nitric oxide levels were similar among the fluid resuscitation groups.

4. DISCUSSION

Research continues into the search for optimal resuscitation fluids that may have added benefit besides their standard use to expand plasma volume and improve hemodynamics. Such fluids would have great utility for combat casualties in austere battlefield environments. In the present study, no one fluid showed a distinct advantage over any other based on hemodynamic or metabolic variables measured, including plasma lactate. That is, oxygen delivery was similar among the groups. However, to reach and maintain a systolic blood pressure of 80 mmHg, the total fluid requirements for LR were over 3-fold greater than for Hextend or PolyHeme. This reflects the expected volume sparing activity of colloids in comparison to crystalloid fluids to achieve the same physiologic response.

The present study also evaluated fluid resuscitation with LR, Hextend and PolyHeme in this model of severe hemorrhage for their potential effects on mediators of signal transduction pathways that may modulate events that lead to the development of systemic inflammatory responses and multiple organ dysfunction. In particular this study focused on protein tyrosine kinase activity, and specifically of the EGF receptor, which has been associated with tissue injury or repair following ischemia/reperfusion type injuries in a number of organs (Majumdar et al, 1996; Branton et al 1998; Yano et al, 1999). Previous studies have shown that activation of EGF-R stimulates a number of genes, which converge on MAP kinases and MAP kinase kinases. After translocation into the nucleus, these cell signals lead to activation of transcription factors such as AP-1 and NF- κ B, which are involved in regulating cell proliferation and the stimulation of cytokines (Xiao et al, 2003). In addition, a study by Imagawa et al (1997) suggested that tyrosine kinase activation had a protective role against ischemia in the rabbit heart. In the present study, duodenal EGF-R concentrations and phosphorylation of EGF-R were maintained near control levels better after hemorrhage by Hextend and PolyHeme, than by LR. These observations might suggest that these fluids may allow for more recovery of the cell to the effects of

Table 1. Indices of Oxidative Stress and Antioxidant Status in Duodenum and Lung from Hemorrhaged Pigs Resuscitated with LR, Hextend or Polyheme¹

	TBARS² (n mol/mg prot)	Antioxidant Potential (n mol/mg prot)	Glutathione (n mol/mg prot)	Nitrate/Nitrite (n mol/mg prot)	8-isoprostane (ng/mg prot)
<u>Duodenum</u>					
Control	0.32±0.01	52±5	1.78±0.08	---	---
LR	0.27±0.01	69±9	1.14±0.07*	12.0±1.4	14.1±1.2
Hextend	0.25±0.04	57±3	1.31±0.12	9.8±0.6	14.8±1.9
Polyheme	0.38±0.05	80±4*	1.31±0.09	9.8±1.0	13.8±0.5
<u>Lung</u>					
Control	0.16±0.04	82±5	1.08±0.10	---	---
LR	0.39±0.03*	56±6*	0.76±0.12	10.2±1.5	27±2
Hextend	0.30±0.02	60±4	1.01±0.11	9.7±1.3	31±3
Polyheme	0.26±0.02*	72±5	1.24±0.11	9.9±1.1	25±2

¹Data expressed as mean ± S.E.

²Thiobarbituric Acid Reactive Substance

*p<0.05 from control

*p<0.05 from LR group

ischemia/reperfusion associated with hemorrhage and fluid resuscitation, when compared with LR, but more work is required to understand more fully the consequences of these results. Similar results were observed in the lung, although the effects of PolyHeme were more pronounced than those of Hextend. As suggested from previous literature in this emerging area, much of this work has focused on tissues of the gastrointestinal tract (Majumdar et al, 1996, Sondeen et al, 1999; Xiao et al, 2003). To our knowledge, this is the first study to expand this field to the lung. Thus, considering the susceptibility of the lung to injury after hemorrhage (McIntyre et al, 2000; Barsness et al, 2004) we recommend continued investigation into the role of protein tyrosine kinases in the response of the lung to hemorrhage and resuscitation. With recent evidence for a role of NF-κB activation in the expression of acute lung injury (Barsness et al, 2004), the possibility of interaction with protein tyrosine kinase pathways seems probable.

At present the specific mechanisms associated with hemorrhage and resuscitation that modulate activation of EGF-R tyrosine kinase activity remain unknown. However, studies have reported that reactive oxygen species generated by an oxidant stress can activate protein tyrosine kinases and inhibit protein phosphatases (Schulze-Osthoff et al, 1997; Kass, 1997). Also, hydrogen peroxide activated EGF-R in vitro (Gamou and Shimizu, 1995). Previous studies have reported that

hemorrhage is associated with generation of reactive oxygen species (Haglund and Gerdin, 1991; Hedlund and Hallaway, 1991). However, the role of reactive oxygen species in the regulation of EGF-R tyrosine kinase activity has been little studied. In a preliminary investigation, we observed that increased duodenal tyrosine kinase activity after hemorrhage and renal ischemia/reperfusion was associated with higher TBARS concentrations in rabbits (Sondeen et al, 1999). The results from the present study also indicated that duodenal and lung glutathione concentrations were better preserved in Hextend and PolyHeme treated animals. In addition, higher lung TBARS concentrations in response to hemorrhage and LR resuscitation were reduced in PolyHeme resuscitated pigs. Further research in this area is necessary to understand more completely the role of reactive oxygen species in signal transduction events associated with the recovery of tissue from ischemia/reperfusion associated with hemorrhage and subsequent fluid resuscitation.

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CONCLUSION

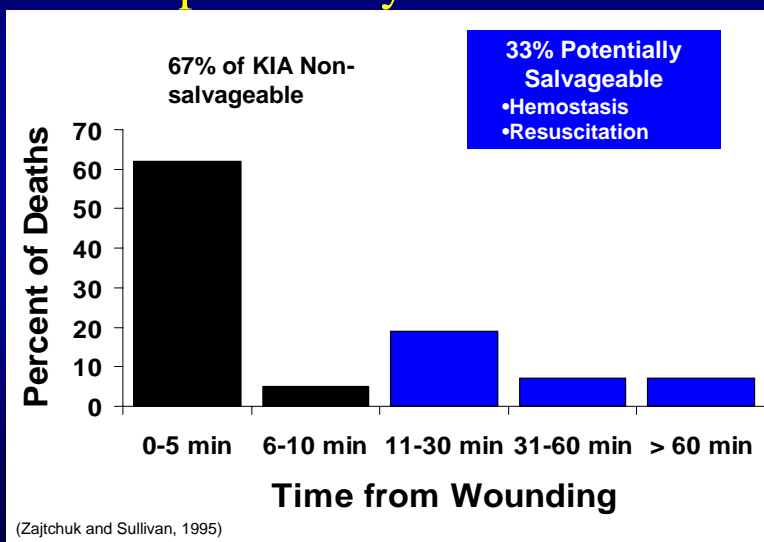
In the present study, standard clinical hemodynamic and metabolic measurements did not detect significant differences among the resuscitation fluids evaluated as to whether one was potentially better than another. However, biochemical analysis revealed differential activation of EGF-R, leading to expressed tyrosine kinase activity in both the duodenum and lung from swine resuscitated with the 3 fluids evaluated and suggesting differences in the degree of induction of the EGF-R signaling pathway. Hextend and PolyHeme were better than LR in restoring both the total and phosphorylated EGF-R concentrations to control levels and PolyHeme seemed the most effective in maintaining antioxidant levels in these tissues. These results indicate that resuscitation fluids used for the treatment of severe hemorrhage can induce biochemical effects that may modulate events related to the development of late complications observed in some resuscitated casualties. Thus, in our efforts to help sustain and improve survival of the Future Force Warrior, evaluation of new resuscitation fluids for treating severe hemorrhage needs to consider their full spectrum of action and not just effects on hemodynamics and global indices of tissue hypoxia.

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Some casualties previously KIA could potentially be saved



Battlefield Constraints

Combat casualty care is constrained by logistics, manpower and the hostile operational environment:

- Limited transportation assets
- Dispersed battlefields
- Long evacuation times
- Limited expertise of medic
- 1st responders must carry all medical treatment equipment, fluids and drugs on their backs
- Noise & unclean conditions
- Confusion of battle



Average Evacuation Times

- | | |
|---------------------------|------------------------|
| • World War I: 18 hr | • Somalia: 12-24 hr |
| • World War II: 8-12 hr | • Desert Storm: 2-4 hr |
| • Korean Conflict: 4-6 hr | • OEF: 12-36 hr |
| • Vietnam: 1-2 hr | |



Future combat scenarios:

- Dispersed, non-linear battlefields, including urban battles
- Longer evacuation times.
- Evacuation of SOF casualties may be delayed up to 72 hr.

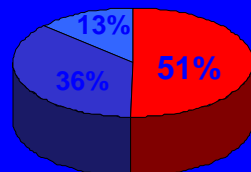


BACKGROUND

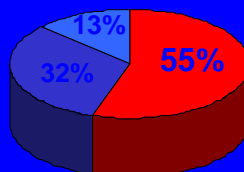
- 50% of battlefield deaths due to acute hemorrhage
- Estimated that 65-80% of casualties need fluid, especially with prolonged evacuation

Predicted causes and mortality with various evacuation times (Bellamy, 1984)

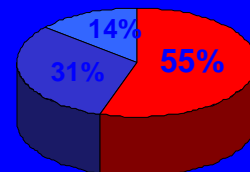
KIA: 19.5%



26%



31.5%



■ Hemorrhage

■ CNS

■ Other

Tactical Field Care

- Initial forward resuscitation determines survival-
Lessons Learned-Falklands

Fluid resuscitation

- Assess for hemorrhagic shock – mental status or absent peripheral pulses
- a. If not in shock:
 - No IV fluids necessary, but PO fluids permissible if conscious
- b. If in shock:
 - Hextend 500 mL IV bolus
 - Repeat once after 30 minutes if still in shock

Hypotensive Resuscitation

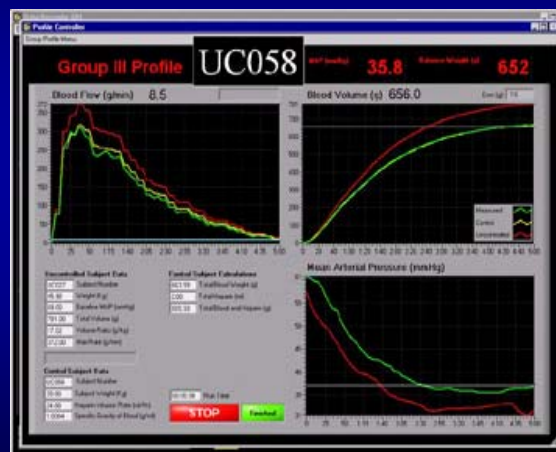
- Resuscitation to pre-hemorrhage blood pressure consumes large volumes of fluid
- Full resuscitation may elevate blood pressure and cause continued bleeding
- Animal experiments suggest that full resuscitation may worsen outcome

Hypotensive Resuscitation

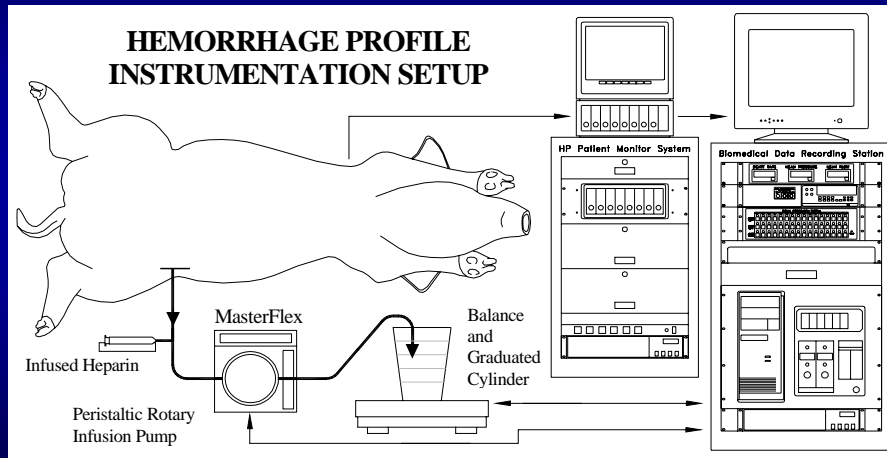
- Advocated for Special Forces using Hextend
 - Resuscitate to detectable radial pulse or mentation
- Potentially solves several problems with battlefield care
 - Limited availability of fluid
 - Lack of surgical hemostasis
- **However:** There is little data available on the consequences of “hypotensive resuscitation” when it is coupled with delayed evacuation

Experimental Design

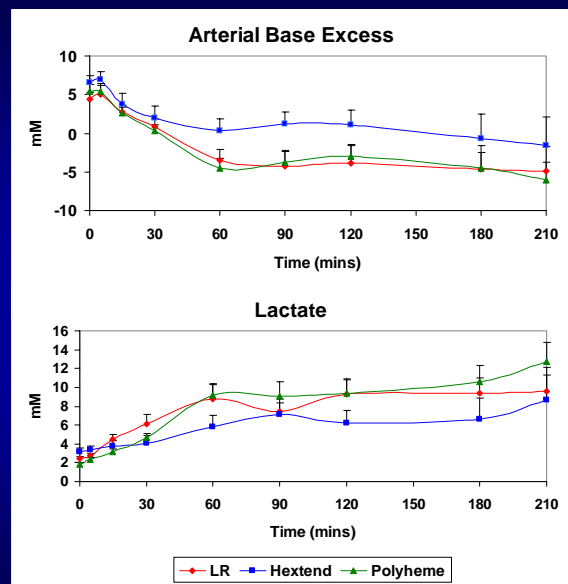
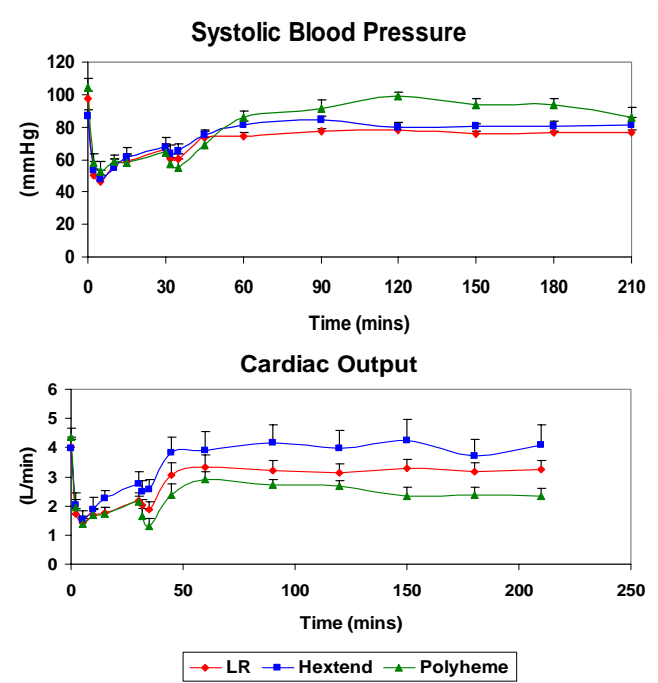
- Pigs hemorrhaged 20 ml/kg
- Second hemorrhage of 8 ml/kg at 30 min that coincided with fluid resuscitation
- Resuscitation continued until SBP of 80 mmHg attained
- Animals monitored for 210 min or until death
- Duodenum and lung evaluated for antioxidant status and activation of the EGF receptor

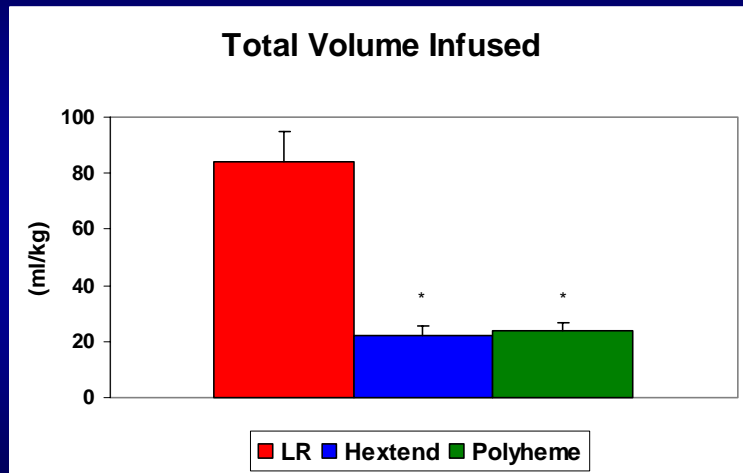


Experimental Design



Hemodynamic and Metabolic Responses

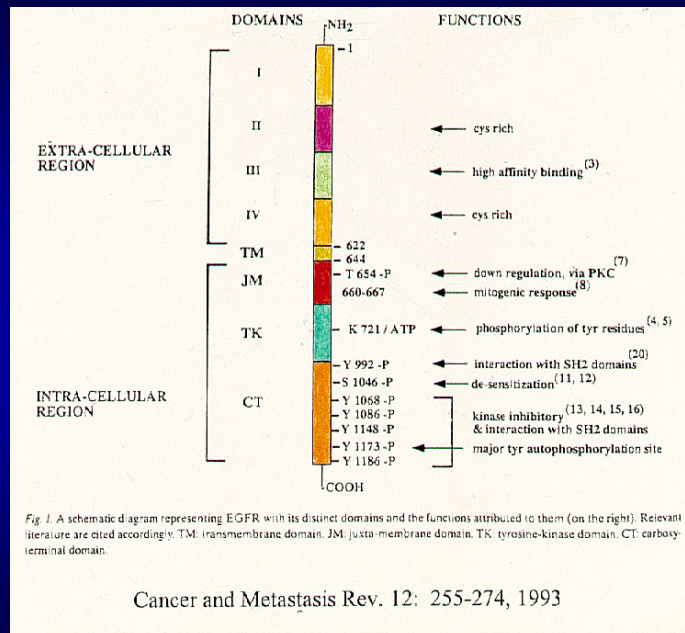




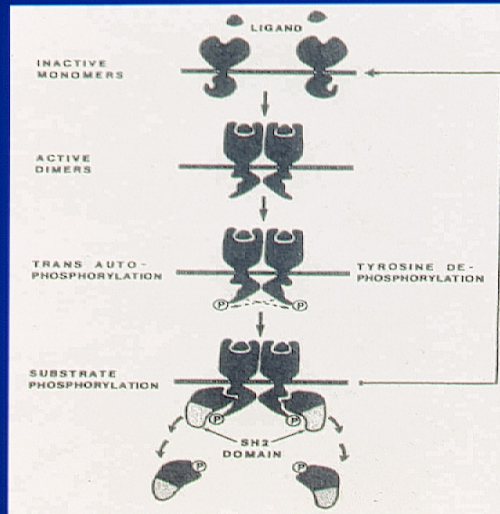
Signal Transduction

Tyrosine Kinases and EGF-R

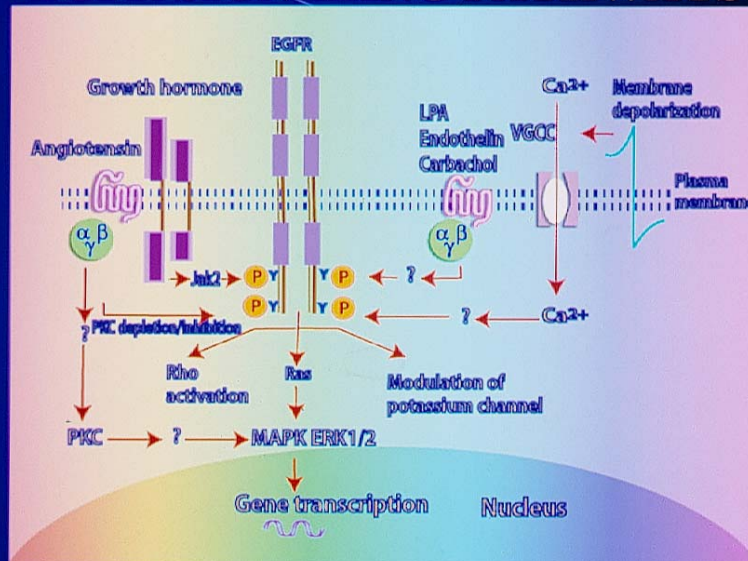
- Activation of protein TK, including EGF-R suggested as early event in signal transduction pathways that leads to synthesis of inflammatory mediators (e.g., cytokines)
- Reactive oxygen species may be involved in mechanism whereby tissue injury stimulates protein TK

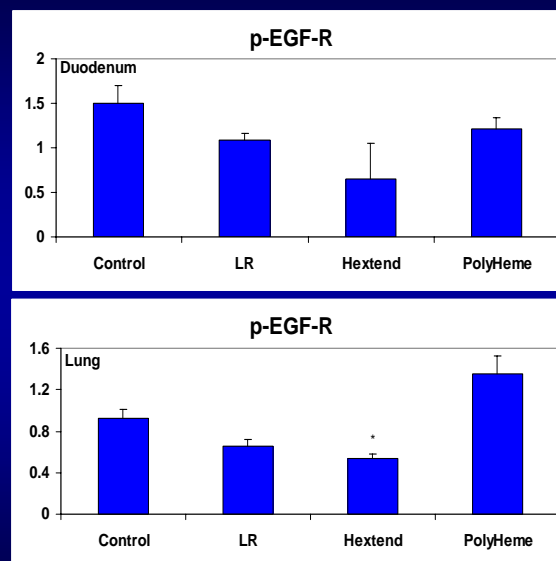
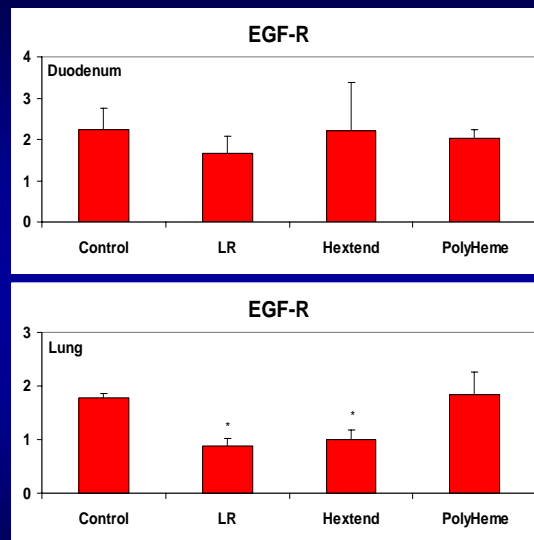


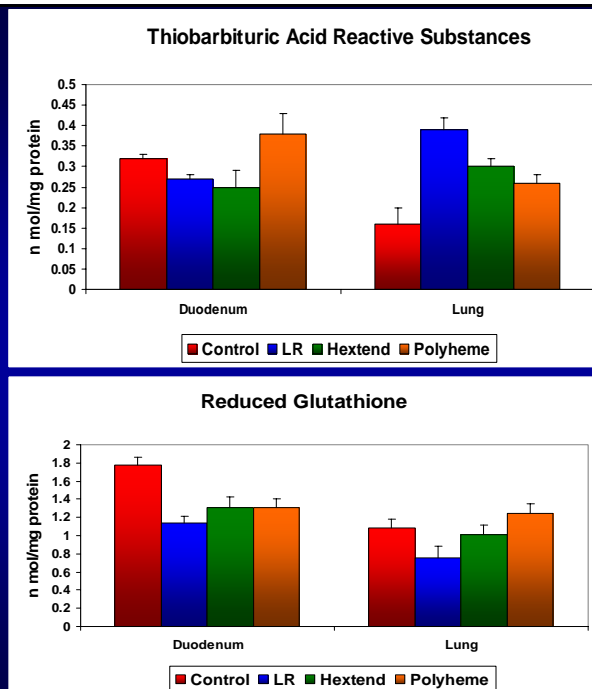
Ligand Binding Induced EGFR Dimerization and Subsequent Activation of Signaling Process



EGFR SIGNALING PATHWAYS







Conclusion

- No fluid evaluated in this study showed clear advantage over any other based on hemodynamic and metabolic variables
- Colloid containing fluids showed volume sparing
- Hemorrhage associated with generation of reactive oxygen species in lung and duodenum
- PolyHeme resuscitation after hemorrhage seemed to maintain EGF-R levels and its phosphorylation closer to control levels than LR or Hextend in both lung and duodenum

Final Comments

- The implication is that PolyHeme may support recovery of the tissue after hemorrhage better than LR or Hextend
- Whether this would translate into reduced development of multi-organ failure is unknown
- These studies are preliminary and further investigation seems warranted

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